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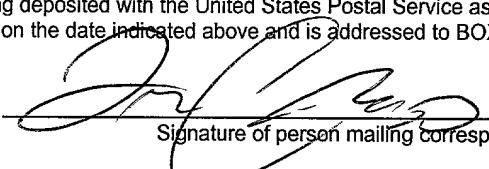
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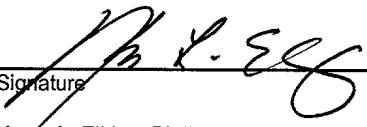

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Substitute Form PTO 1390 U.S. Department of Commerce Patent and Trademark Office		Attorney's Docket Number: 50125/011001	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. Application Number:	
INTERNATIONAL APPLICATION NUMBER		INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/DE98/02896		September 23, 1998	September 23, 1997 May 11, 1998
TITLE OF INVENTION:		COSTIMULATING POLYPEPTIDE OF T CELLS, MONOCLONAL ANTIBODIES, AND THE PREPARATION AND USE THEREOF	
APPLICANTS FOR DO/EO/US:		Richard Kroczek	
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
1.	<input checked="" type="checkbox"/>	This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.	
2.	<input type="checkbox"/>	This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.	
3.	<input checked="" type="checkbox"/>	This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).	
4.	<input checked="" type="checkbox"/>	A proper Demand for International Preliminary Examination was made by the 19 th month from the earliest claimed priority date.	
5.	<input checked="" type="checkbox"/>	A copy of the International Application as filed (35 U.S.C. 371(c)(2)).	
a.		is transmitted herewith (required only if not transmitted by the International Bureau).	
b.	<input checked="" type="checkbox"/>	has been transmitted by the International Bureau.	
c.		Is not required, as the application was filed with the United States Receiving Office (RO/US).	
6.	<input checked="" type="checkbox"/>	A translation of the International Application into English (35 U.S.C. 371(c)(2)).	
7.	<input checked="" type="checkbox"/>	Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).	
a.		are transmitted herewith (required only if not transmitted by the International Bureau).	
b.		have been transmitted by the International Bureau.	
c.		have not been made; however, the time limit for making such amendments has NOT expired.	
d.	<input checked="" type="checkbox"/>	have not been made and will not be made.	
8.		A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).	
9.		An oath or declaration of the inventors (35 U.S.C. 371(c)(4)).	

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10.	A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).												
11.	An Information Disclosure Statement under 37 CFR 1.97 and 1.98.												
12.	An assignment for recording. A separate cover sheet in compliance with 37 3.28 and 3.31 is included.												
13.	A FIRST preliminary amendment.												
	A SECOND or SUBSEQUENT preliminary amendment.												
14.	A substitute specification.												
15.	A change of power of attorney and/or address letter.												
16.	Other items or information:												
17. X	<p>The following fees are submitted:</p> <p>BASIC NATIONAL FEE (37 CFR 1.492(A)(1)-(5)):</p> <table> <tr> <td>Search Report has been prepared by the EPO or JPO</td> <td>\$ 930.00</td> </tr> <tr> <td>International preliminary examination fee paid to USPTO (37 CFR 1.482)</td> <td>\$ 720.00</td> </tr> <tr> <td>No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee (37 CFR 1.445(a)(2)) paid to USPTO</td> <td>\$ 690.00</td> </tr> <tr> <td>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO</td> <td>\$ 1070.00</td> </tr> <tr> <td>International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)</td> <td>\$ 98.00</td> </tr> </table>		Search Report has been prepared by the EPO or JPO	\$ 930.00	International preliminary examination fee paid to USPTO (37 CFR 1.482)	\$ 720.00	No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	\$ 690.00	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO	\$ 1070.00	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	\$ 98.00	
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Surcharge of \$130 for furnishing the oath or declaration later than <input type="checkbox"/> 20 OR <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		\$											
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE										
Total claims	56 - 20 = 36		x \$22.00	\$ 792.00									
Independent claims	3 - 3 = 0		x \$82.00	\$									
Multiple dependent claims (if applicable)			+ \$270.00	\$ 270.00									
TOTAL OF ABOVE CALCULATIONS =		\$2,132.00											
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed with this request (Note 37 CFR 1.9, 1.27, 1.28).		\$											
SUBTOTAL =		\$ 2,132.00											
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 OR <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).		+	\$										
TOTAL NATIONAL FEE =		\$ 2,132.00											
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property.		+	\$										
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		Amount to be refunded	\$
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a.	X	A check in the amount of \$ 2,132.00 to cover the above fees is enclosed.	
b.		Please charge my Deposit Account No. 03-2095 in the amount of \$ [**.**] to cover the above fees.	
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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.			
SEND ALL CORRESPONDENCE TO:		 Signature Karen L. Elbing, Ph.D. Reg No. 35,238	
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Revised: 17 March 2000

Bundesrepublik Deutschland

17. März 2000

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5 **Costimulating polypeptide of T cells, monoclonal anti-
 bodies, and the preparation and use thereof**

The invention relates to a polypeptide (8F4 molecule) having the biological activity of costimulating T cells. The invention further relates to monoclonal antibodies against the 8F4 molecule and hybridoma cells which produce the monoclonal antibodies. The invention additionally relates to the use of substances which inhibit the biological activity of the polypeptide 8F4 according to the invention, in particular monoclonal antibodies, natural or synthetic ligands, agonists or antagonists, as pharmaceuticals. In particular, the invention relates to the use of these substances for the prevention or therapy of disorders in which the immune system is involved, in particular for the treatment of autoimmune diseases and for the prevention of rejection reactions with organ transplants. The invention additionally relates to the use of the 8F4 molecule or of cells which contain the 8F4 molecule as pharmaceuticals, in particular for the prevention or therapy of disorders in which the immune system is involved, in particular for the treatment of cancers, Aids, asthmatic disorders or chronic viral diseases such as HCV or HBV infections. The invention further relates to the use of substances which specifically recognize the polypeptide according to the invention, in particular monoclonal antibodies, natural or synthetic ligands, agonists or antagonists, for the diagnosis of disorders in which the immune system is involved. In particular, the invention relates to diagnosis by means of an ELISA detection, a flow cytometry or a Western blot, a radioimmunological detection, a nephelometry or a histochemical staining.

T lymphocytes recognize their antigen, which is presented by "antigen-presenting cells", for example dendritic cells, B cells and macrophages, through their T-cell receptor. Recognition of the antigen by the T-cell receptor alone is, however, in most cases insufficient for adequate activation of T lymphocytes. The latter makes additional simultaneous stimulation (also called "costimulation" hereinafter) by other receptor molecules on the surface of the T lymphocytes necessa-

ry. One of these receptor molecules is the so-called CD28 receptor which is stimulated by the costimulating molecule B7. If these "costimulatory" molecules, for example CD28, are effective, then the activation of the T cell reaches an adequate level after recognition of the antigen by the T-cell receptor. After such a complete activation, the T cell expresses additional molecules, for example CD25, CD69, CD71, on the surface and synthesizes numerous cytokines, for example IL-2 and 10 IFN- γ , which function as messengers. Both these additional surface molecules and the cytokines serve for the T cell to exchange information with other cells in the immune system. The activated T cells direct the entire 15 antigen-specific immune defences through the additional surface molecules and the cytokines. Both the generation of cytotoxic cells ("killer cells") and the generation of antigen-specific antibodies by B cells is controlled in this way. Cytotoxic cells, as well as the 20 specifically formed antibodies, eliminate viral or bacterial pathogens which enter the body. In some cases, however, the immune response goes too far, and the immune system is directed against the body's own cells. This leads to the occurrence of "autoimmune diseases", 25 for example to rheumatoid arthritis, ankylosing spondylitis, Sjögren's syndrome, ulcerative colitis inter alia. One of the essential sites of cooperation between 30 antigen-activated T cells and other cells of the immune system are the secondary lymphatic organs, including the tonsils. This is where the T lymphocytes are activated by the antigen presented by dendritic cells, and this is where T lymphocytes interact with B cells. Through this interaction, B cells secrete, after several intermediate stages of differentiation, antigen-specific antibodies of the IgM and IgG types.

35 The costimulatory molecule which has been characterized best and is among the most effective to date is the CD28 surface molecule (called CD28 receptor or CD28 hereinafter) which is constitutively expressed on a large fraction of T cells. Costimulation by CD28 40 *in vitro* leads, after recognition of the antigen by the T-cell receptor, to a very large increase in cytokine secretion, for example of IL-2 and IFN- γ , and to a marked up-regulation of the expression of cell surface molecules such as CD25, CD69, CD71, which are necessary 45 for interaction of T cells with other immune cells, for example B lymphocytes; cf. Chambers and Allison, *Current Opinion in Immunology* 9 (1997), 396-404. Costimulation via the CD28 receptor can also markedly increase the proliferation of T lymphocytes. In addition, costi-

mulation via the CD28 receptor optimizes the T-cell control of B-lymphocyte function so that there is increased secretion of antibodies.

If the function of the CD28 receptor is abolished, there is a drastic loss of function in the immune defences. This has been shown by means of a transgenic mouse in which the CD28 gene was destroyed by homologous recombination (a so-called "CD28 knock-out"). The destruction in this way of activation of the antigen-specific T cells leads to lack of costimulation. This in turn leads to a disturbance of T-cell function, that is to say to a reduced proliferation of T cells and to a drastically reduced synthesis of various cytokines. The lack of costimulation eventually leads to a reduced function of the antigen-specific immune defences. Thus, *inter alia*, the formation of antigen-specific IgG1 and IgG2 antibodies by B lymphocytes is reduced to 10% of the normal level through the lack of CD28; cf. Shahinian et al., *Science* 262 (1993), 609-612; Lucas et al. *Journal of Immunology* 154 (1995), 5757-5768. It is also possible *in vitro* to prevent the Aids virus entering T lymphocytes by costimulation by CD28; cf. Riley et al., *Journal of Immunology* 158 (1997), 5545-5553. Corresponding experiments *in vivo* have not yet been carried out. It is known that CD28 switches on many cytokine genes which may lead to considerable side effects *in vivo*. Blockade of CD28 receptors by a soluble CTLA-4 immunoglobulin molecule has been employed successfully in a monkey model to prevent the rejection of transplanted kidneys. In this case, CTLA-4 had been employed in combination with an antibody against the CD40 ligand molecule; cf. Kirk et al., *Proc. Natl. Acad. Sci. USA* 94 (1997) 8789-8794. However, blockade of CD28 receptors affects all T lymphocytes and not just those already activated because CD28 is constitutively expressed on T lymphocytes.

There is thus a need for a costimulating surface molecule which is expressed only on activated T lymphocytes. The invention is therefore based on the object of providing a surface molecule on activated T cells which has a strong costimulatory effect on central functions of T lymphocytes. Another object of the invention is to provide substances, for example monoclonal antibodies against the costimulatory surface molecule, natural or synthetic ligands, agonists or antagonists of the surface molecule.

In a first embodiment, the invention relates to a polypeptide having the biological activity of co-

stimulation of T cells, characterized in that a) the polypeptide occurs on activated CD4⁺ and CD8⁺ T lymphocytes but not on resting or activated B cells, granulocytes, monocytes, NK cells (natural killer cells) or dendritic cells, and b) the polypeptide is a dimer, the polypeptide having a molecular weight of about 55 to 60 kDa (kilodalton) determined in a non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and the two polypeptide chains of the polypeptide having a molecular weight of about 27 kDa and about 29 kDa measured in a reducing SDS-PAGE.

The polypeptide according to the invention (also called 8F4 molecule or 8F4 hereinafter) is expressed only after activation of the T lymphocytes, specifically both on CD4⁺ and on CD8⁺ T cells. In a non-reducing SDS-PAGE, the 8F4 molecule has a molecular weight between about 55 and 60 kDa (kilodalton). The 8F4 molecule is composed of two peptide chains, and the two peptide chains have a molecular weight of about 27 and about 29 kDa in a reducing SDS-PAGE. The 8F4 antigen can be unambiguously detected histologically on activated T lymphocytes in the lymphatic tissue of the tonsils and lymph nodes, especially in the germinal centres, the site of interaction of T lymphocytes and B lymphocytes in the generation of antibodies. Tonsillar T cells isolated *ex vivo* are about 50-80% positive for the 8F4 antigen and show signs of advanced activation. The 8F4 molecule is not detectable on resting or activated B cells, granulocytes, monocytes, NK cells and dendritic cells.

An important biological activity of the 8F4 molecule is its costimulating activity on T lymphocytes. The costimulating activity can be determined by the method of Linsley et al., *Journal of Experimental Medicine* 176 (1992), 1595-604. The costimulating activity of the 8F4 molecule resembles the costimulating activity of the CD28 molecule, which has been identified as the central enhancement element of antigen recognition by the immune system. The 8F4 molecule differs in many aspects from CD28, however. Thus, expression of the 8F4 molecule on the surface of the T cells requires induction, whereas CD28 is constitutively expressed. There are also distinct differences detectable in the function: costimulation by CD28 leads to overexpression of numerous lymphokines, *inter alia* of interleukin-2 (IL-2). Costimulation by 8F4 also leads to enhanced secretion of lymphokines, but not of IL-2. The costimulatory activity of the 8F4 molecule thus differs from the activity of the CD28 molecule.

Since stimulation by 8F4 does not switch on all cytokine genes, costimulation by 8F4 *in vivo* is advantageous, for example compared with costimulation via the CD28 receptor. Moreover, the induction, the expression, the site of expression and the function of the 8F4 molecule differ from all other known molecules with costimulatory activity.

The 8F4 molecule according to the invention is a novel surface molecule on activated T cells which has a strong costimulatory effect on central functions of T lymphocytes. Expression *in vivo* indicates inter alia an essential involvement of the 8F4 molecule in the cooperation of T cells with other cells of the immune system such as B cells or dendritic cells within the humoral and cellular immune defences against viruses and bacteria.

After expression, the 8F4 molecule has *in vitro* a strong costimulatory effect on various functions of T lymphocytes:

- 20 1. Marked enhancement of the proliferation of T lymphocytes.
2. Marked enhancement of the synthesis of certain cytokines by T lymphocytes.
- 25 3. Greatly increased expression of control molecules, for example surface molecules and cytokines, on and in T lymphocytes.
4. Marked improvement in T-cell-induced antibody formation (IgM and IgG) by B cells.

The present invention furthermore provides a polypeptide having the biological activity of costimulation of T cells and having an amino acid sequence which shows at least 40% homology with the sequence comprising 199 amino acids in Fig. 15 (SEQ ID NO:2), or a biologically active fragment or an analogue thereof. A biologically active fragment or analogue is a fragment or analogue which likewise shows a costimulatory effect on T-cell lymphocytes or at least displays a biological effect of the nature of a blockage. Preference is given to a polypeptide or a biologically active fragment or analogue thereof which shows at least 60% homology with the sequence comprising 199 amino acids in Fig. 15 (SEQ ID NO:2). In a particularly preferred embodiment, the polypeptide according to the invention comprises an amino acid sequence which shows at least 80% homology with the sequence comprising 199 amino acids in Fig. 15

(SEQ ID NO:2), or a biologically active fragment or analogue thereof.

5 A particularly preferred polypeptide has the biological activity of costimulation of T cells and comprises an amino acid sequence as shown in Fig. 15 (SEQ ID NO:2), or a biologically active fragment or an analogue thereof.

10 The invention includes allelic variants, fragments and analogues of the 8F4 molecule. These variants include naturally occurring allelic variants, substitution analogues in which one or more amino acids have been substituted by different amino acids, substitution analogues in which one or more amino acids have been substituted by different amino acids, deletion 15 analogues in which one or more amino acids have been deleted and addition analogues in which one or more amino acids have been added. Deletion and addition of one or more amino acids may be done either at an internal region of the polypeptide or at the amino or carboxyl terminus.

20 Polypeptides according to the invention fused to heterologous polypeptides are likewise embraced.

25 In another embodiment, the invention relates to DNA sequences which encode a polypeptide according to the invention or a biologically active fragment or analogue thereof.

30 These DNA sequences include the sequence shown in SEQ ID NO:1 (Fig. 16) as well as allelic variants, fragments, and analogues having biological activity.

A preferred DNA sequence encodes a polypeptide having the biological activity of costimulation of T cells, the sequence being selected from the group consisting of:

35 a) the DNA sequence shown in SEQ ID NO:1 (Fig. 16) and its complementary strand

b) DNA sequence hybridizing with the sequences in (a) and

c) DNA sequences which, because of the degeneracy of 40 the genetic code, hybridize with the sequences in (a) and (b). The aforementioned DNA sequences preferably hybridize together under stringent conditions.

45 Also provided are vectors which comprise these DNA sequences, and host cells which are transformed or transfected with these vectors.

In another embodiment, the invention relates to monoclonal antibodies against the 8F4 molecule. The monoclonal antibodies according to the invention can be prepared in a conventional way by the method described by Milstein and Köhler, *Nature* 256 (1975), 495-497. In particular, the monoclonal antibodies according to the invention can be prepared by immunizing mice with T cells which have been activated *in vitro* with phorbol myristate acetate (PMA) and ionomycin ("2-signal system") for 24 h. The spleen cells of the immunized mice are fused with myeloma cells. 8F4-specific monoclonal antibodies are identified by their recognition of 2-signal-activated but not resting T lymphocytes. Moreover 8F4-specific antibodies do not stain T cells stimulated with one signal (either PMA or ionomycin) in a detection method carried out in a conventional way. 8F4-specific antibodies produce a typical staining pattern of tonsillar T cells and recognize an antigen of about 55 to 60 kDa in a non-reducing SDS-PAGE and of about 27 kDa and about 29 kDa in a reducing SDS-PAGE on activated T lymphocytes.

In another embodiment, the invention relates to hybridoma cells which produce the monoclonal antibodies according to the invention.

In another embodiment, the invention relates to the use of substances which inhibit the biological activity of the polypeptide 8F4 according to the invention as pharmaceuticals. The use of the monoclonal antibodies according to the invention, natural or synthetic ligands, agonists or antagonists of the 8F4 molecule is particularly preferred. These substances can be used as pharmaceuticals for the prevention or therapy of disorders in which the immune system is involved, in particular for the treatment or autoimmune diseases or for prevention of rejection reactions in organ transplants. Blockade of the interaction of the 8F4 antigen with its receptor improves, for example, the prevention of organ rejection because such a blockade affects only previously activated T lymphocytes. Another embodiment of the invention relates to the use of the polypeptide according to the invention as pharmaceutical. The polypeptide according to the invention can be used in particular for the prevention or therapy of disorders in which the immune system is involved, in particular for the treatment of cancers, AIDS, asthmatic disorders or chronic viral diseases such as HCV or HBV infections.

The polypeptide according to the invention can likewise be introduced into cells in a conventional

way so that these cells for example constitutively express the polypeptide. For example, the nucleic acid sequence encoding the polypeptide or a vector comprising the nucleic acid sequencing encoding the polypeptide, for example the cDNA or genomic DNA, promoters, enhancers and other elements required for expression of the nucleic acid sequence can be inserted into a cell. The 8F4 cDNA (2641 nucleotides) depicted in Fig. 16 (SEQ ID NO:1) or fragments or derivatives thereof, is preferably employed for expression of the polypeptide according to the invention or fragments thereof.

The polypeptide according to the invention can also be introduced for example by means of liposomes into cells which then form the polypeptide on their cell surface. These cells can be used as pharmaceuticals according to the invention, in particular for restoring correct regulation of the human immune system, as occurs within the framework of numerous chronic infectious diseases, for example within the framework of AIDS, asthmatic disorders or in chronic viral hepatitis (for example HCV, HBV), or for stimulating the immune system *in vitro* or *in vivo* such as, for example, be used for the therapy of cancers.

In another embodiment, substances which specifically recognize the polypeptide according to the invention are used for diagnosing disorders in which the immune system is involved, the substances embracing in particular a monoclonal antibody, natural or synthetic ligands, agonists or antagonists. It is possible to use for the diagnosis for example an ELISA detection, flow cytometry, Western blot, radioimmunoassay, nephelometry or a histochemical staining. The substances which recognize the polypeptide according to the invention also comprise nucleic acid sequences, the latter preferably being employed for hybridization and/or nucleic acid (RNA, DNA) amplification (for example PCR).

In another embodiment, the invention relates to substances which have a positive or negative effect on (modulate) the signal transduction pathway of the polypeptide according to the invention into the T cell, and to the use of these substances as pharmaceuticals.

In another embodiment, the invention relates to substances which prevent up-regulation of the polypeptide according to the invention on the T-cell surface, and to the use thereof as pharmaceuticals.

In another embodiment, the polypeptide according to the invention or fragments thereof is expressed by a transgenic animal.

5 In another embodiment, the invention embraces a transgenic animal in which the gene which codes for the polypeptide according to the invention has been switched off ("knock-out").

The figures serve to illustrate the invention:

10 Fig. 1 shows the result of an immunoprecipitation of the 8F4 antigen from activated human T cells. (a) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; 12% polyacrylamide gel (PAA gel)) reducing, (b) SDS-PAGE (10% PAA gel) non-reducing. The 15 conditions for elution of the antigen from the 8F4 matrix are indicated. "SDS" means sodium dodecyl sulphate; "DTT" means dithiothreitol, "Mr" means molecular weight and "kDa" means kilodalton.

20 Fig. 2a shows the result of a flow cytometry after induction of the 8F4 antigen on CD4⁺ T cells. The activation time for the T cells is indicated in parentheses. "PMA" means phorbol myristate acetate; "PHA" means phytohaemagglutinin; "OKT3" is a monoclonal antibody against CD3; "MLR" means mixed lymphocyte reaction; "mAK 9.3" is a monoclonal antibody against CD28; 25 "SEB" means staphylococcal enterotoxin B.

30 Fig. 2b shows the result for the kinetics of induction of the 8F4 antigen on CD4⁺ T cells after activation with PMA and ionomycin in a flow cytometry. The immunofluorescence (log) is plotted against the cell count.

35 Fig. 3 shows the result of a flow cytometry for identifying molecules which are involved in the induction of 8F4 in the mixed lymphocyte reaction. "bio" means biotinylated antibody.

Fig. 4 shows the result of a histochemical investigation for localization of 8F4-positive cells in the tonsil.

40 Fig. 5 shows the result of an expression analysis of 8F4 on T and B cells from human tonsils in a flow cytometry. "bioPE" means biotinylated antibody and streptavidin-phycoerythrin secondary reagent.

45 Fig. 6 shows the coexpression of the 8F4 molecule with other activation markers (CD69, CD45) in a flow cytometry.

Fig. 7 shows diagrammatically the enhanced expression of activation molecules on T lymphocytes after costimulation by 8F4. Open circles (O) represent 8F4 antibodies; triangles (◆) represent nonspecific antibodies of the same isotype; filled circles (●) represent anti-CD28 antibodies-9.3.

Fig. 8 shows a diagrammatic comparison of the costimulating effect of 8F4 with the costimulating effect of CD28. "mAk" means monoclonal antibodies; "ATAC" means "activation induced T-cell-derived and chemokine-related"; "cpm" means radioactive disintegrations per minute.

Fig. 9 shows diagrammatically the enhancement of the synthesis of the antibodies of the IgM and IgG types by B cells after costimulation of T cells. "ng" means nanogram; "ml" means millilitre; "mAk" means monoclonal antibody.

Fig. 10 shows diagrammatically the prevention of the activation-induced apoptosis of peripheral T cells after costimulation by 8F4.

Fig. 11 shows expression of the 8F4 antigen on the MOLT-4V cell line. MOLT-4V cells were stained with a fluorescein-labelled 8F4 antibody (8F4-FITC) and investigated in flow cytometry (unfilled line, comparing with an isotype control (filled line)).

Fig. 12 shows the two-dimensional gel electrophoresis. A MOLT-4V cell lysate from 300×10^6 cells was immunoprecipitated as described. The eluate was fractionated on a non-reducing SDS-PAGE (10% PAA), and the region around 60 kDa was cut out of the gel. To reduce the disulphide bridges in the 8F4 molecule, the piece of gel was incubated in 5.3 M urea, 0.5 M Tris, pH 8.0, 1% SDS, 1% β -mercaptoethanol at 50°C for 1 h, and the free cysteine residues in the molecule were alkylated with 10 mM iodoacetamide (Sigma, Deisenhofen) (37°C, 30 min). The piece of gel was equilibrated in 1xSDS-PAGE sample buffer for a further 30 min and mounted on a 12% PAA-SDS gel (with stacking gel). After fractionation by electrophoresis, the gel underwent silver staining. The location of the 8F4 protein was determined by surface iodination (cf. Fig. 1) and is marked by a circle. (All the procedures not described in detail were carried out by standard methods, see, for example, Westermeier, R., Electrophoresis in Practice, VCH Verlagsgesellschaft, Weinheim, 1997).

Fig. 13 shows a hybridization with Oligo 1 (SEQ ID NO:3). Lambda clones immobilized on nitrocellu-

lose filters were hybridized with Oligo 1 as described in the examples. Exposure on an X-ray film is depicted (detail).

Fig. 14 shows a Northern blot analysis with the 8F4 cDNA. Hybridization of a Northern blot with the 8F4 cDNA produces a band which migrates in the gel between the 18S and 28S RNA. Fig. 14A shows the behaviour as 2-signal-dependent (see above) activation antigen: no expression in resting lymphoid cells (PBL), strong expression in PMA+ionomycin-activated CD4+ T cells and distinctly reduced expression with PMA or ionomycin alone. Fig. 14B shows the strength of mRNA expression after different stimulation times (T cells (purified via nylon wool adherence, NTC), stimulated with PMA+ionomycin). Besides this the MOLT-4 cell lines (ATCC CRL-1582) which shows only minimal expression, and on the far right the MOLT-4V which was used for the cloning and which shows a distinct signal. Also loaded is the RNA from other cell lines on which no 8F4 expression was detectable in the analysis by flow cytometry: CEM (ATCC CCL-119), HUT-102 (ATCC TIB-162), HUT-78 (ATCC TIB-161), Jurkat (ATCC TIB-152), DG75 (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) ACC83), Karpas 299 (Fischer, P. et al. (1988), Blood, 72:234-240), DEL (Barbey, S. et al. (1990), Int. J. Cancer, 45:546-553).

Fig. 15 shows the amino acid sequence of the polypeptide 8F4 (SEQ ID NO:2).

Fig. 16 shows the 8F4 cDNA (SEQ ID NO:1).

The following examples illustrate the invention and are not to be understood restrictively.

Example 1: Generation of the 8F4 antibody

Balb/c mice were immunized with human T cells which had previously been activated for 24 h with 33 ng/ml of the phorbol ester phorbol myristate acetate (PMA) (Sigma, Deisenhofen) and with 200 ng/ml of the Ca^{2+} ionophore ionomycin (Sigma, Deisenhofen) (so-called "2-signal activation"). After boosting three times, the spleen cells of the mice were fused with the myeloma P3X63Ag8.653 (ATCC No. CRL-1580), and antibody-secreting hybridomas were generated by standard methods; cf. Peters and Baumgarten, Monoclonal Antibodies, Springer, Heidelberg, 1992. The resulting antibodies were screened for activated versus resting T cells

in flow cytometry. Activated ("2-signal activation") and resting T cells were incubated with the hybridoma supernatant and then labelled with a fluorescence-labelled secondary antibody; cf. Shapiro, Practical
5 Flow Cytometry, Wiley-Liss, New York, 1995. Only the antibodies which recognize molecules which were induced exclusively by PMA and the Ca^{2+} ionophore ionomycin on the T-cell surface, but not by one of the agents alone ("2-signal molecules"), were selected for further purification.
10 The resulting antibodies were investigated in flow cytometry for similarity to or difference from known antibodies against activation molecules (cf. Table 1) on T cells. The criteria for this were, besides the abovementioned "2-signal dependence", the kinetics of induction on stimulated T cells and the expression
15 on various cell lines.

Example 2: Immunoprecipitation of the 8F4 antigen

20 Surface molecules from activated human T cells were iodinated with ^{125}I by standard methods and immunoprecipitated with the antibody 8F4 by standard methods; cf. Goding, Monoclonal Antibodies: Principle and Practice, Academic Press, London, 1996. The antibody for
25 the immunoprecipitation was coupled by the method of Schneider et al., *Journal of Biological Chemistry* 257 (1982), 10766-10769, to protein G (Pharmacia, Freiburg) (8F4 matrix). The matrix was washed as described by Schneider et al., see above. The immunoprecipitated 8F4
30 molecule was analysed for its molecular mass in an SDS-PAGE (non-reduced and reduced) in a conventional way; Goding, see above.

Example 3: Flow cytometry

35 The 8F4-carrying T cells were analysed in flow cytometry by standard methods; cf. Shapiro, Practical Flow Cytometry, Wiley-Liss, New York, 1995.

Exemplary embodiment 3.1: Flow cytometry after induction of the 8F4 antigen on CD4^+ T cells.

CD4⁺ T cells from peripheral blood were stimulated with various agents in a conventional way, and investigated for expression of the 8F4 molecule in flow cytometry by a conventional method. The activation time for the T cells was between 24 hours and 144 hours with the various agents. Modes of activation: phorbol myristate acetate (PMA; 33 ng/ml), ionomycin (200 ng/ml), phytohaemagglutinin (PHA 1.5 mg/ml), OKT3 (monoclonal antibody against CD3), mixed lymphocyte reaction (MLR, between 50,000 CD4⁺ T cells and 100,000 B cells), mAk 9.3 (monoclonal antibody against CD28), staphylococcal enterotoxin B (SEB, 0.1 ng/ml). Analysis revealed that various stimuli are suitable for inducing the 8F4 molecule on T cells, but the expression density differs. The most potent stimuli, besides the highly active pharmacological agents PMA and ionomycin, are those which represent a costimulatory situation such as, for example, accessory cells in the MLR or the costimulating mAk 9.3.

Exemplary embodiment 3.2: Kinetics of induction of the 8F4 antigen on CD4⁺ T cells after activation with PMA and ionomycin.

CD4⁺ T cells from peripheral blood were stimulated with PMA (33 ng/ml) and ionomycin (200 ng/ml) in a conventional way and investigated after 0, 4, 8, 12, 24 and 48 hours for expression of the 8F4 molecule by flow cytometry in a conventional way. The molecule is detectable on the surface after only four hours, and thus belongs to the class of relatively early activation antigens. There is still good expression of the antigen even after 48 hours.

Exemplary embodiment 3.3: Flow cytometry to identify molecules which are involved in the induction of 8F4 in the "mixed lymphocyte reaction".

while the 8F4-negative cells carry the phenotype CD45RA. CD45RA is mainly expressed by so-called "naïve" T cells, whereas CD45RO is associated with an effector cell function. The 8F4⁺ cells are thus mainly "mature" T cells. CD45RO and CD45RA are isoforms of CD45.

Example 4: Localization of 8F4-positive cells in the tonsil

Tonsillar tissue in frozen sections was stained with the 8F4 antibody in the APAAP technique (alkaline phosphatase-anti-alkaline phosphatase) by standard methods. 8F4⁺ cells were found preferentially in the germinal centre of the tonsils, but also in part in the T-cell zone of the tonsils.

Example 5: Costimulation of T lymphocytes

96-well plates were coated with a goat anti-mouse Ig antibody (20 µg/ml), washed, and loaded with the anti-CD3 monoclonal antibody OKT3 (various dilutions of an ascites) and the 8F4 antibody according to the invention (2 µg/ml). The OKM1 antibody or the 2A11 antibody (both 2 µg/ml) were used as isotype control.

Exemplary embodiment 5.1: Enhanced expression of activation molecules on T lymphocytes after costimulation by 8F4.

Purified CD4⁺ T cells from peripheral blood were activated with various concentrations of the monoclonal antibody OKT3 and, at the same time, costimulated with the 8F4 antibody or a nonspecific antibody of the same isotype. As comparison, costimulation was carried out with the anti-CD28 antibody-9.3, one of the strongest known costimulatory antibodies. Even with optimal stimulation by CD3, a costimulatory effect is still to be seen both with the mAk 8F4 and with the mAk 9.3. In the suboptimal OKT3 region, that is to say the region in which complete T-cell activation cannot be achieved

without costimulation, both antibodies are able to increase the expression of other activation antigens by a factor of 4 to 100, and the effect of the anti-CD28 antibody is still visible even at very high OKT3 dilutions. This is attributable to the fact that with very weak OKT3 stimulation the 8F4 antigen is no longer brought to the cell surface and thus cannot be crosslinked by the mAk 8F4 either.

Exemplary embodiment 5.2: Comparison of the costimulating effect of 8F4 with the costimulating effect of CD28.

Purified CD8⁺ T cells were stimulated with a suboptimal concentration of the monoclonal antibody OKT3 for 51 h. The costimulators employed were antibody 15 8F4, antibody 9.3 (anti-CD28) and isotype controls (2 µg/ml each). After completion of the stimulation time, the T-cell proliferation rate was determined by ³H-thymidine incorporation. In parallel cultures, the supernatant was removed and the concentration of the 20 cytokines ATAC/lymphotoxin and IL-2 was determined. 8F4 and CD28 differ greatly from one another in relation to IL-2 synthesis. CD28 costimulation leads, as also described in the prior art (Chambers and Allison, *Current Opinion in Immunology* 9 (1997), 396-404), to 25 very extensive IL-2 secretion. By contrast, IL-2 production with 8F4 is below the detection limit. However, proliferation is comparable in the two mixtures, and thus the autocrine growth of the T cells must be attributed to other factors on costimulation of 8F4. The 30 two antibodies also differ scarcely at all in the costimulatory effect in relation to secretion of the lymphokine ATAC.

Example 6: Determination of the immunoglobulins synthesized by B cells after interaction with 8F4-costimulated T cells

96-well plates were coated with a goat anti-mouse Ig antibody (20 µg/ml), and loaded with the anti-CD3 monoclonal antibody OKT 3 (1:500 to 1:80,000 ascites) and the 8F4 antibody according to the invention 5 (2 µg/ml). The OKM1 antibody or the 2A11 antibody was used as isotype control. In some experiments, a costimulation was carried out with a CD28-specific antibody ("9.3") for comparison; cf. Hara et al., *Journal of Experimental Medicine* 161 (1985), 1513-1524. 10 50,000 purified (Magnetobeads, Dynal, Hamburg) CD4⁺ T cells (>95% purity) from peripheral blood and 25,000 allogeneic tonsillar B cells (negative selection by T-cell rosetting with sheep erythrocytes, 96% purity) were pipetted into each well of the culture plates pretreated 15 in this way, and cocultivated for 8 days. After this period, the supernatant was removed and analysed for the concentration of secrete immunoglobulins of the IgM and IgG types in an ELISA in a conventional way; cf. Nishioka and Lipsky, *Journal of Immunology* 153 (1994), 20 1027-1036.

Exemplary embodiment 6.1: Enhancement of the synthesis of antibodies of the IgM and IgG types by the B cells after costimulation of T cells.

Purified CD4⁺ T cells from peripheral blood were 25 cocultivated with allogeneic B cells from tonsils for 8 days in a conventional way. With suboptimal stimulation of the T cells with the OKT3 antibody, the costimulation of the T cells by 8F4 enhances the secretion of IgM and IgG immunoglobulins by a factor of 40.

30 Example 7: Prevention of the activation-induced apoptosis of peripheral T cells after costimulation by 8F4.

Peripheral T cells (purified by nylon wool adherence in a conventional way), were stimulated with PHA (1.5 mg/ml) for 20 h and cultivated with IL-2 for 35 6 days. The cells were then restimulated by OKT3 with

and without costimulation by mAk 8F4 (2 μ g/ml). The apoptosis was determined by staining the DNA with propidium iodide in flow cytometry (FACS). With suboptimal stimulation via the T-cell receptor complex,
5 costimulation by 8F4 can reduce the proportion of apoptotic cells by a factor of 4.

Example 8: Cloning of the cDNA coding for the 8F4 protein

10 A cell line (MOLT-4V) which expresses the 8F4 antigen constitutively was identified in flow cytometry by staining with a fluorescent dye-coupled 8F4 antibody (Fig. 11). The MOLT-4V line is a variant of the human T-cell line MOLT-4 (American Type Culture Collection (ATCC) CRL-1582).

15 This cell line was used for preparative purification of the 8F4 antigen with the aid of the monoclonal antibody:

20 The cells were cultivated on a large scale (150 l) in roller culture bottles and removed by centrifugation, and the cellular proteins were extracted using a lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF (Sigma, Deisenhofen), 1% NP-40 (Boehringer, Mannheim)). Cell nuclei and other insoluble constituents were removed by ultracentrifugation. The cell lysate obtained in this way was preincubated with Sepharose CL4-B (Pharmacia, Freiburg) for 2 h in order to remove proteins which bind nonspecifically to Sepharose. Incubation then took place with the 8F4 immunoaffinity matrix described in Example 2 above (4 h at
25 4°C). The matrix was packed into a column and then washed several times under conditions with which there is exclusive removal of nonspecifically binding proteins (1.50 mM Tris, pH 8.0, 300 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.5% NP-40; 2.50 mM Tris, pH 8.0, 150 mM
30 NaCl, 1 mM EDTA, 1 mM PMSF, 0.5% NP-40, 0.1% SDS; 3.

0.2 M glycine pH 4.0, 0.5% CHAPS (Merck, Darmstadt)). The 8F4 antigen was eluted from the matrix with 0.2 M glycine, pH 2.5, 0.5% CHAPS. The eluate was concentrated by ultrafiltration (Amicon Centricon 10, Millipore, Eschborn).

In order to achieve further purification of the 8F4 molecule, the dimeric structure of the molecule (see Fig. 1) was utilized in a two-dimensional gel electrophoresis (nonreducing/reducing): since most proteins occur as monomer, they migrate on a diagonal in gel electrophoresis, whereas the 8F4 molecule migrates at 55-60 kDa in the 1st dimension (nonreducing) and at 27 and 29 kDa (Fig. 12) in the 2nd dimension (reducing).

For preparative fractionation, the immunoprecipitates from in each case 20×10^9 cells were prepared as described above for Fig. 12 and fractionated in two-dimensional gel electrophoresis, the gel was stained with Coomassie blue G250 (Biorad, Munich) and the areas indicated in Fig. 12 were separately cut out of the gel (8F4-27 kDa and 8F4-29 kDa respectively).

For peptide microsequencing, the proteins from in each case 4 pieces of gel were digested with trypsin and eluted from the gel. The tryptic fragments were fractionated by HPLC and individual fractions were subjected to Edman degradation (method described in detail in Groettrup, M. et al. (1996), Eur. J. Immunol., 26:863-869).

Sequencing of the 8F4-29 kDa sample revealed, besides fragments of known proteins, a peptide sequence XRLTDVT for which no human correlate was found in any of the protein databases.

Unambiguous translation back of a protein sequence into a DNA sequence is not possible. Thus,

translation of the above peptide sequence back into an oligonucleotide with 17 nucleotides results in 2048 permutations. However, a specific method (Wozney, J.M. (1990), Methods Enzymol. 182:738-751) makes it 5 possible to screen a cDNA bank with degenerate oligonucleotides. On the basis of the peptide sequence found, 2 oligonucleotides (Oligo 1 (SEQ ID NO:3); MGN CTS ACN GAY GTN AC, 512 permutations; Oligo 2 (SEQ ID NO:4): MGN YTD ACN GAY GTN AC, 1024 permutations) were synthesized.
10

For screening, a cDNA bank was constructed from the MOLT-4V cell line also used for the protein purification:

Complete RNA was isolated by the guanidinium/CsCl 15 method (Chirgwin, J.M. et al. (1979), Biochemistry 18:5294-5299), and mRNA was concentrated on Oligo-dT-cellulose columns (Gibco BRL, Eggenstein). Synthesis of the first and second cDNA strands was carried out using a commercial cDNA synthesis system (Gibco BRL, Eggenstein) 20 using Oligo-dT primers in accordance with the manufacturer's instructions. The cDNA was ligated via EcoRI adaptors into the Lambda ZAPII vector (Stratagene, Heidelberg).

The cDNA bank was plated out by standard methods (Vogeli, G. and Kaytes, P.S. (1987), Methods Enzymol., 152:407-515) and the Lambda DNA was immobilized 25 on nitrocellulose filters (Optitran BA-S 85, Schleicher & Schuell, Dassel).

The abovementioned oligonucleotides were radio- 30 labelled using T4 polynucleotide kinase (NEBL, Schwabach) and γ -³²P ATP (NEN Du Pont, Brussels) (Wallace, R.B. and Miyada, C.G. (1987), Methods Enzymol., 152:432-442).

Hybridization of the filters took place in a buffer described for degenerate oligonucleotides (Wozney, J.M. (1990), Methods Enzymol. 182:738-751) with 3 M tetramethylammonium chloride (Roth, Karlsruhe) at 48°C. The filters were washed as described in the abovementioned reference, the washing temperature being 50°C. Exposure of these filters on an X-ray film revealed about 50 positive clones per 100,000 plated phages (Fig. 13).

6 clones were further characterized by transferring them by in vivo excision, using the method described by the manufacturer of the vector (Stratagene, Heidelberg), into a plasmid vector, and partially sequencing with T3 and T7 primers (BigDye Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, USA). One of the clones contained a sequence which on translation provided exactly the peptide sequence which was sought. This clone was used for hybridization of a Northern blot (Fig. 14) (Kroczek, R.A. (1993), J. Chromatogr., 618, 133-145). The expression pattern of the mRNA corresponded exactly to the expression of the 8F4 molecule as was known from investigations on the monoclonal antibody by flow cytometry. Since the clone which was found contained only the 3' end of the cDNA sought, a fragment on the 5' side was used to isolate the complete 8F4 cDNA. Several clones were sequenced on both strands.

The 8F4 cDNA (2641 nucleotides) is depicted in Fig. 16 and in the sequence listing under SEQ ID NO:1, and codes for a protein having 199 amino acids (Nucleotides 68-664), depicted in Fig. 15 and in the sequence listing under SEQ ID NO:2. Sequencing of several independent clones from the cDNA bank showed some deviations from the sequence shown here, but these are all in the 3'-untranslated region:

Pos. 909-910:deletion

Pos. 1631:T->C

Pos. 2074:G->T

Pos. 2440:G->C

5 Pos. 2633: alternative polyadenylation site

Table 1:

Table 1 summarizes the antibodies used (clone), their source of origin (source), the specificity for their particular antigen (specificity) and, where appropriate, their labelling (label).

Specificity	Label	Isotype	Clone	Source
CD3	Cy-Chrome	IgG1	UCHT1	Pharmingen, Hamburg
CD3	-	IgG2a	OKT3	ATCC CRL-8001
CD11b	-	IgG2b	OKM1	ATCC CRL-8026
CD25	FITC	IgG2a	B1.49.9	Immunotech, Hamburg
CD28	-	IgG2a	9.3	Immunex Corp., Seattle
CD45RA	Cy-Chrome	IgG2b	HI100	Pharmingen, Hamburg
CD45RO	FITC	IgG2a	UCHL1	Immunotech, Hamburg
CD69	FITC	IgG1	FN50	Pharmingen, Hamburg
CD80	-	IgG1	L307.4	Becton Dickinson, Heidelberg
CD86	-	IgG2b	IT2.2	Pharmingen, Hamburg
CD154	FITC	IgG1	TRAP-1	Hybridoma ¹
MHCII	-	IgG2a	L243	ATCC HB-55
8F4	-	IgG1	8F4	Hybridoma ¹
8F4	Biotin	IgG1	8F4	Hybridoma ¹
Isotype IgG1	-	IgG1	2A11	Hybridoma ^{1,2}
Isotype IgG1	FITC	IgG1	2A11	Hybridoma ^{1,2}
Isotype IgG1	Biotin	IgG1	ASA-1	Hybridoma ¹

¹ The hybridoma cell line was generated in a conventional way, and the antibody was purified and labelled where appropriate.

² Directed against a synthetic peptide

5

The antisera and secondary reagents used in the examples were purchased from: goat anti-mouse Ig, FITC conjugated, from Jackson Immuno Research Lab., USA; Streptavidin, PE-conjugated, from Jackson Immuno Research Lab., USA; rabbit anti-mouse Ig fraction, from Sigma, Deisenhofen.

It will be apparent to those skilled in the art that various modifications and variations can be made to the compositions and processes of this invention. Thus, it is intended that the present invention cover such modifications and variations, provided they come within the scope of the appended claims and their equivalents.

20 Priority applications DE 19741929.1 filed September 23, 1997 and DE 19821060.4 filed May 11, 1998 including the specification, drawings, claims and abstract, are hereby incorporated by reference. All publications cited herein are incorporated in their entireties by
25 reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

5 (A) NAME: Federal Republic of Germany, ultimately represented by the Director
of the Robert-Koch-Institut
10 (B) STREET: Nordufer 20
(C) CITY: Berlin
(D) STATE OR
PROVINCE: Berlin
(E) COUNTRY: Germany
(F) POSTAL CODE: 13353

15 (ii) TITLE OF INVENTION: Costimulating polypeptide of T cells,
monoclonal antibodies, and the preparation and use thereof

20 (iii) NUMBER OF SEQUENCES: 4

25 (iv) COMPUTER-READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

30 (v) CURRENT APPLICATION DATA:
APPLICATION NUMBER:

(2) INFORMATION FOR SEQ ID NO: 1:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2641 base pairs
(B) TYPE: Nucleotides
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGAGAGCCTG AATTCACTGT CAGCTTGAA CACTGAACGC GAGGACTGTT AACTGTTTCT	60
GGCAAACATG AAGTCAGGCC TCTGGTATTT CTTTCTCTTC TGCTTGCAGCA TTAAAGTTTT	120
AACAGGAGAA ATCAATGGTT CTGCCAATTA TGAGATGTTT ATATTTACAA ACGGAGGTGT	180
ACAAATTATA TGCAAATATC CTGACATTGT CCAGCAATTAA AAAATGCAGT TGCTGAAAGG	240
GGGGCAAATA CTCTGCAGTC TCACTAAGAC AAAAGGAAGT GGAAACACAG TGTCCATTAA	300
GAGTCTGAAA TTCTGCCATT CTCAGTTATC CAACAAACAGT GTCTCTTTTT TTCTATACAA	360
CTTGGACCAT TCTCATGCCA ACTATTACTT CTGCAACCTA TCAATTTTG ATCCCTCTCC	420
TTTAAAGTA ACTCTTACAG GAGGATATTT GCATATTTAT GAATCACAAC TTTGTTGCCA	480
GCTGAAGTTC TGGTTACCCA TAGGATGTGC AGCCTTGTT GTAGTCTGCA TTTTGGGATG	540
CATACTTATT TGTTGGCTTA CAAAAAGAA GTATTCACTCC AGTGTGCACG ACCCTAACGG	600

TGAATACATG TTCAAGAGAG CAGTGAACAC AGCCAAAAAA TCTAGACTCA CAGATGTGAC 660
CCTATAATAT GGAACCTCTGG CACCCAGGCA TGAAGCACGT TGGCCAGTTT TCCTCAACTT 720
GAAGTGCAAG ATTCTCTTAT TTCCGGGACC ACGGAGAGTC TGACTTAACT ACATACATCT 780
TCTGCTGGTG TTTTGTCAA TCTGGAAGAA TGACTGTATC AGTCAATGGG GATTTTAACA 840
GACTGCCTTG GTACTGCCGA GTCCTCTCAA AACAAACACC CTCTTGCAAC CAGCTTGGA 900
GAAAGCCCAG CTCTGTGTG CTCACTGGGA GTGGAATCCC TGTCTCCACA TCTGCTCCTA 960
GCAGTGCACTC AGCCAGTAAACACAT TTACAAGAAA AATGTTTAA AGATGCCAGG 1020
GGTACTGAAT CTGCAAAGCA AATGAGCAGC CAAGGACAG CATCTGTCCG CATTCACTA 1080
TCATACTACC TCTTCTTCT GTAGGGATGA GAATTCCCTCT TTTAATCAGT CAAGGGAGAT 1140
GCTTCAAAGC TGGAGCTATT TTATTCCTGA GATGTTGATG TGAACTGTAC ATTAGTACAT 1200
ACTCAGTACT CTCCTTCAAT TGCTGAACCC CAGTTGACCA TTTTACCAAG ACTTTAGATG 1260
CTTTCTTGTG CCCTCAATT TCTTTTAA AATACTTCTA CATGACTGCT TGACAGCCCA 1320
ACAGCCACTC TCAATAGAGA GCTATGTCTT ACATTCTTTC CTCTGCTGCT CAATAGTTT 1380
ATATATCTAT GCATACATAT ATACACACAT ATGTATATAA AATTCTATAAT GAATATATT 1440
GCCTATATTC TCCCTACAAG AATATTTTG CTCCAGAAAG ACATGTTCTT TTCTCAAATT 1500
CAGTTAAAT GGTTTACTTT GTTCAAGTTA GTGGTAGGAA ACATTGCCCG GAATTGAAAG 1560
CAAATTTATT TTATTTATCCT ATTTTCTACC ATTATCTATG TTTTCATGGT GCTATTAATT 1620
ACAAGTTTAG TTCTTTTGT AGATCATATT AAAATTGCAA ACAAAATCAT CTTAATGGG 1680
CCAGCATTCT CATGGGGTAG AGCAGAATAT TCATTTAGCC TGAAAGCTGC AGTTACTATA 1740
GGTTGCTGTC AGACTATACC CATGGTGCCT CTGGGCTTGA CAGGTCAAAA TGGTCCCCAT 1800
CAGCCTGGAG CAGCCCTCCA GACCTGGGTG GAATTCCAGG GTTGAGAGAC TCCCTGAGC 1860
CAGAGGCCAC TAGGTATTCT TGCTCCCAGA GGCTGAAGTC ACCCTGGAA TCACAGTGGT 1920
CTACCTGCAT TCATAATTCC AGGATCTGTG AAGAGCACAT ATGTGTCAGG GCACAATTCC 1980
CTCTCATAAA AACCACACAG CCTGGAAATT GGCCCTGGCC CTTCAAGATA GCCTTCTTAA 2040
GAATATGATT TGGCTAGAAA GATTCTAAA TATGTGGAAT ATGATTATTC TTAGCTGGAA 2100
TATTTCTCT ACTTCCGTGTC TGCTGCCA AGGCTCTGA AGCAGCCAAT GTCGATGCAA 2160
CAACATTGT AACTTTAGGT AACTGGGAT TATGTTGTAG TTTAACATT TGTAACTGTG 2220
TGCTTATAGT TTACAAGTGA GACCCGATAT GTCATTATGC ATACTTATAT TATCTTAAGC 2280
ATGTGTAATG CTGGATGTGT ACAGTACAGT ACTGAACCTG TAATTGAAAT CTAGTATGGT 2340
GTTCTGTTT CAGCTGACTT GGACAACCTG ACTGGCTTGTG CACAGGTGTT CCCTGAGTTG 2400
TTTGCAGGTT TCTGTGTGTG GGGTGGGGTA TGGGGAGGAG AACCTTCATG GTGGCCACC 2460
TGGCCTGGTT GTCCAAGCTG TGCCCTGACA CATCCTCATC CCCAGCATGG GACACCTCAA 2520

GATGAATAAT AATTCAACAAA ATTTCTGTGA AATCAAATCC AGTTTTAAGA GGAGCCACTT 2580

ATCAAAGAGA TTTAACAGT AGTAAGAAGG CAAAGAATAA ACATTTGATA TTCAGCAACT 2640

G 2641

(2) INFORMATION FOR SEQ ID NO: 2:

5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 199 amino acids
- (B) TYPE: Amino acid
- (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Lys Ser Gly Leu Trp Tyr Phe Phe Leu Phe Cys Leu Arg Ile Lys
1 5 10 15

Val Leu Thr Gly Glu Ile Asn Gly Ser Ala Asn Tyr Glu Met Phe Ile
20 25 30

Phe His Asn Gly Gly Val Gln Ile Leu Cys Lys Tyr Pro Asp Ile Val
35 40 45

Gln Gln Phe Lys Met Gln Leu Leu Lys Gly Gly Gln Ile Leu Cys Asp
50 55 60

Leu Thr Lys Thr Lys Gly Ser Gly Asn Thr Val Ser Ile Lys Ser Leu
65 70 75 80

Lys Phe Cys His Ser Gln Leu Ser Asn Asn Ser Val Ser Phe Phe Leu
85 90 95

Tyr Asn Leu Asp His Ser His Ala Asn Tyr Tyr Phe Cys Asn Leu Ser
100 105 110

Ile Phe Asp Pro Pro Pro Phe Lys Val Thr Leu Thr Gly Gly Tyr Leu
115 120 125

His Ile Tyr Glu Ser Gln Leu Cys Cys Gln Leu Lys Phe Trp Leu Pro
130 135 140

Ile Gly Cys Ala Ala Phe Val Val Val Cys Ile Leu Gly Cys Ile Leu
145 150 155 160

Ile Cys Trp Leu Thr Lys Lys Tyr Ser Ser Ser Val His Asp Pro
165 170 175

Asn Gly Glu Tyr Met Phe Met Arg Ala Val Asn Thr Ala Lys Lys Ser
180 185 190

Arg Leu Thr Asp Val Thr Leu
195

(2) INFORMATION FOR SEQ ID NO: 3:

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: Nucleotides
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(iii) HYPOTHETICAL: Yes
10 (iv) ANTISENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

MGNCTSACNG AYGTNAC 17

15 (2) INFORMATION FOR SEQ ID NO: 4:

20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: Nucleotides
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(iii) HYPOTHETICAL: Yes
25 (iv) ANTISENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

MGNYTDACNG AYGTNAC 17

Bundesrepublik Deutschland (brd)

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5

Patent claims

1. A costimulating molecule

a) having the biological activity of costimulation of T cells,

10 b) which occurs on activated CD4⁺ and CD8⁺ T lymphocytes but not resting or activated B cells, granulocytes, monocytes, NK cells or dendritic cells, and

15 c) which has two polypeptide chains, the said molecule having a molecular weight of about 55 to 60 kDa determined in a nonreducing SDS polyacrylamide gel electrophoresis, and the two polypeptide chains of the said molecule having a molecular weight of about 27 kDa and about 29 kDa measured in a reducing SDS polyacrylamide gel electrophoresis.

20 2. A costimulating molecule having the biological activity of costimulation of T cells comprising an amino-acid sequence which shows at least 40% homology with the sequence comprising 199 amino acids in Fig. 15 (SEQ ID NO:2), or a biologically active fragment or an

25 analogue thereof.

3. A costimulating molecule having the biological activity of costimulation of T cells according to Claim ✓ 2 and comprising the amino acid sequence shown in Fig. 15 (SEQ ID NO:2), or a biologically active fragment or 30 an analogue thereof.

4. A DNA sequence which encodes a costimulating molecule according to Claim 1 or a fragment thereof.

5. A DNA sequence which encodes a costimulating molecule according to Claim 2 or a fragment thereof.

6. A DNA sequence encoding a costimulating molecule having the biological activity of costimulation of T cells, the sequence being selected from the group consisting of:

5 a) the DNA sequence shown in SEQ ID NO:1 (Fig. 16) and its complementary strand

b) DNA sequence hybridizing with the sequences in (a) and

10 c) DNA sequences which, because of the degeneracy of the genetic code, hybridize with the sequences in (a) and (b).

7. A plasmid or a viral DNA vector comprising a DNA sequence according to Claim 4.

15 8. A plasmid or a viral DNA vector comprising a DNA sequence according to Claim 5.

9. A prokaryotic or eukaryotic host cell stably transformed or transfected with a plasmid or DNA vector according to Claim 4.

20 10. A prokaryotic or eukaryotic host cell stably transformed or transfected with a plasmid or DNA vector according to Claim 5.

11. Method for preparing a costimulating molecule according to Claim 1, comprising the cultivation of the 25 host cell according to Claim 9 for expression of the said molecule in the host cell.

12. Method for preparing a costimulating molecule according to Claim 1, comprising the cultivation of the host cell according to Claim 10 for expression of the 30 said molecule in the host cell.

13. Method for preparing a costimulating molecule according to Claim 2, comprising the cultivation of the

host cell according to Claim 9 for expression of the said molecule in the host cell.

14. Method for preparing a costimulating molecule according to Claim 2, comprising the cultivation of the 5 host cell according to Claim 10 for expression of the said molecule in the host cell.

15. An antibody which binds a costimulating molecule according to Claim 1.

16. An antibody which binds a costimulating molecule 10 according to Claim 2.

17. An antibody according to Claim 15, which is a monoclonal antibody.

18. An antibody according to Claim 16, which is a monoclonal antibody.

15 19. A monoclonal antibody which specifically recognizes a costimulating molecule according to Claim 1, characterized in that B cells of mice which are immunized with human T lymphocytes activated PMA and the Ca^{2+} ionophore ionomycin are fused with a myeloma cell 20 line to give an antibody-secreting hybridoma, and the monoclonal antibodies are purified in flow cytometry for 2-signal molecule-activated against resting T cells.

25 20. A monoclonal antibody which specifically recognizes a costimulating molecule according to Claim 2, characterized in that B cells of mice which are immunized with human T lymphocytes activated PMA and the Ca^{2+} ionophore ionomycin are fused with a myeloma cell line to give an antibody-secreting hybridoma, and the 30 monoclonal antibodies are purified in flow cytometry for 2-signal molecule-activated against resting T cells.

21. A hybridoma cell which generates the monoclonal antibody according to Claim 15.

22. A hybridoma cell which generates the monoclonal antibody according to Claim 16.

23. Use of substances which inhibit the biological activity of a costimulating molecule according to Claims 1 as pharmaceuticals.

24. Use of substances which inhibit the biological activity of a costimulating molecule according to Claims 2 as pharmaceuticals.

10 25. Use according to Claim 23, where the substances comprise a monoclonal antibody, natural or synthetic ligands, agonists or antagonists.

15 26. Use according to Claim 24, where the substances comprise a monoclonal antibody, natural or synthetic ligands, agonists or antagonists.

20 27. Use of substances which inhibit the biological activity of a costimulating molecule according to Claim 1 for the production of a pharmaceutical for the treatment of autoimmune diseases, for the prevention of rejection reactions in organ transplants and for the treatment of dysregulation of the immune system.

25 28. Use of substances which inhibit the biological activity of a costimulating molecule according to Claim 2 for the production of a pharmaceutical for the treatment of autoimmune diseases, for the prevention of rejection reactions in organ transplants and for the treatment of dysregulation of the immune system.

30 29. Use of a costimulating molecule according to Claim 1 as pharmaceuticals.

30 30. Use of a costimulating molecule according to Claim 2 as pharmaceuticals.

31. Use of a costimulating molecule according to Claim 1 for the production of pharmaceuticals for the

treatment of cancers, Aids, asthmatic disorders and chronic viral diseases such as HCV and HBV infections.

32. Use of a costimulating molecule according to Claim 2 for the production of pharmaceuticals for the treatment of cancers, Aids, asthmatic disorders and chronic viral diseases such as HCV and HBV infections.

33. Use of cells comprising a costimulating molecule according to Claim 1 as pharmaceuticals.

34. Use of cells comprising a costimulating molecule according to Claim 2 as pharmaceuticals.

35. Use of cells according to Claim 33 for the production of a pharmaceutical for the treatment of cancers, Aids, asthmatic disorders and chronic viral diseases such as HCV and HBV infections.

36. Use of cells according to Claim 34 for the production of a pharmaceutical for the treatment of cancers, Aids, asthmatic disorders and chronic viral diseases such as HCV and HBV infections.

37. Use of substances which specifically recognize a costimulating molecule according to Claim 1 for the diagnosis of disorders in which the immune system is involved.

38. Use of substances which specifically recognize a costimulating molecule according to Claim 2 for the diagnosis of disorders in which the immune system is involved.

39. Use according to Claim 37, where the substances comprise nucleic acid (RNA, DNA) molecules.

40. Use according to Claim 38, where the substances comprise nucleic acid (RNA, DNA) molecules.

41. Use according to Claim 37, where a hybridization or nucleic acid application technique (for example PCR) is used for the diagnosis.

42. Use according to Claim 38, where a hybridization or nucleic acid application technique (for example PCR) is used for the diagnosis.

5 43. Use according to Claim 37, where the substances comprise a monoclonal antibody, natural and synthetic ligands, agonists and antagonists.

44. Use according to Claim 38, where the substances comprise a monoclonal antibody, natural and synthetic
10 ligands, agonists and antagonists.

45. Use according to Claim 37, where an ELISA detection, flow cytometry, Western blot, radioimmunoassay, nephelometry and a histochemical staining is used for the diagnosis.

15 46. Use according to Claim 38, where an ELISA detection, flow cytometry, Western blot, radioimmunoassay, nephelometry and a histochemical staining is used for the diagnosis.

20 47. Use of substances which have a positive or negative effect on (modulate) the signal transduction pathway of a costimulating molecule according to Claim 1 into the T cell as pharmaceuticals.

25 48. Use of substances which have a positive or negative effect on (modulate) the signal transduction pathway of a costimulating molecule according to Claim 2 into the T cell as pharmaceuticals.

49. Use of substances which prevent the up - regulation of a costimulating molecule according to Claim 1 on the T-cell surface as pharmaceuticals.

30 50. Use of substances which prevent the up - regulation of a costimulating molecule according to Claim 2 on the T-cell surface as pharmaceuticals.

51. Use of a costimulating molecule according to Claim 1 for producing antibodies.

52. Use of a costimulating molecule according to Claim 2 for producing antibodies.

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Sample: 100 μg of protein from 100 μl of 10% sucrose gradient supernatant

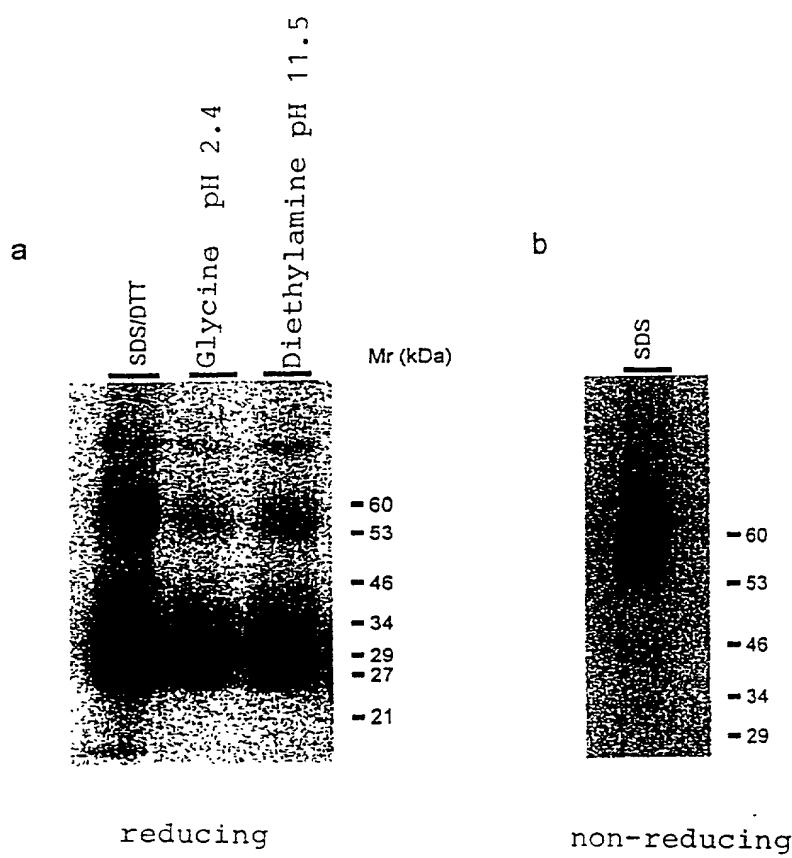


FIG. 1

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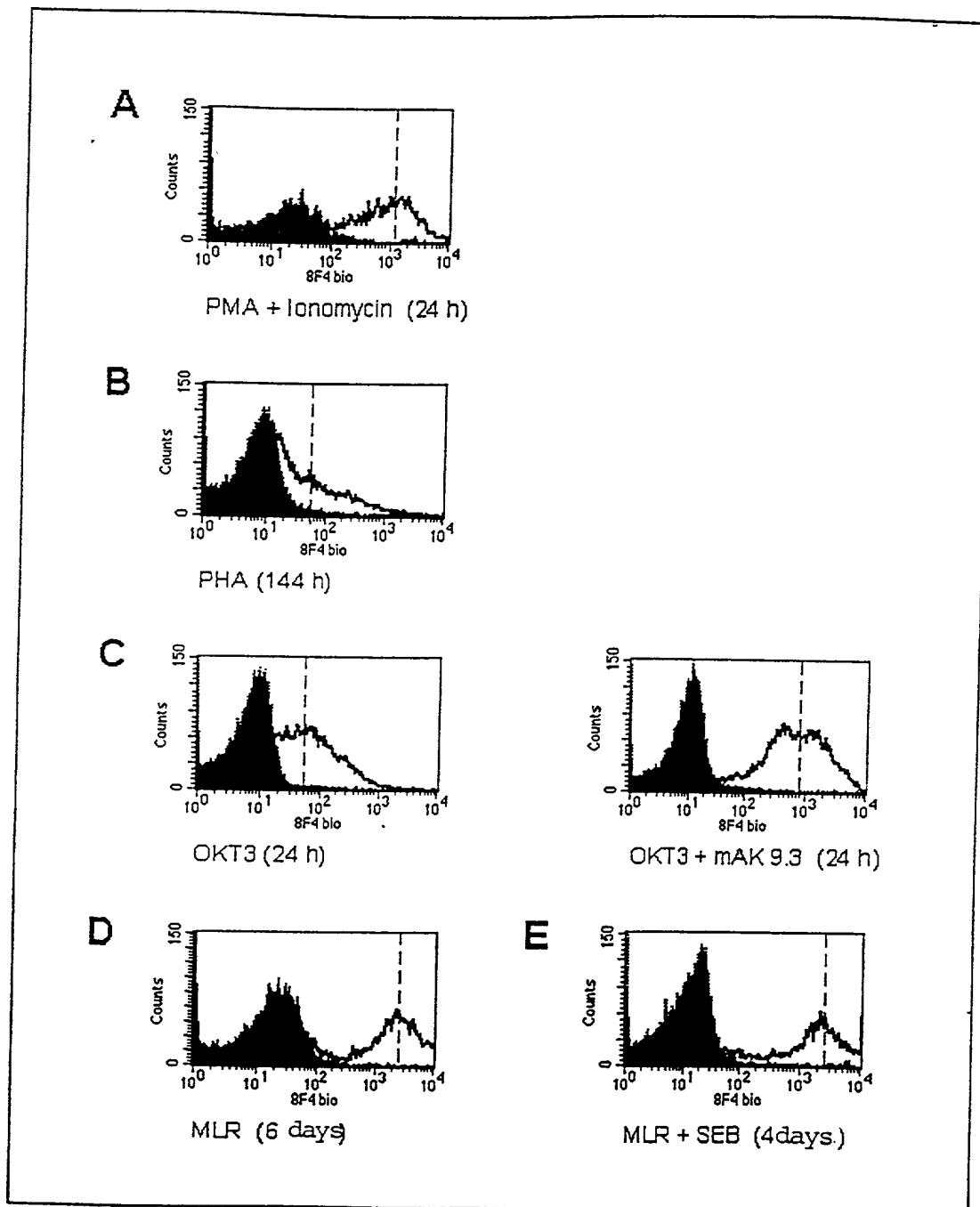
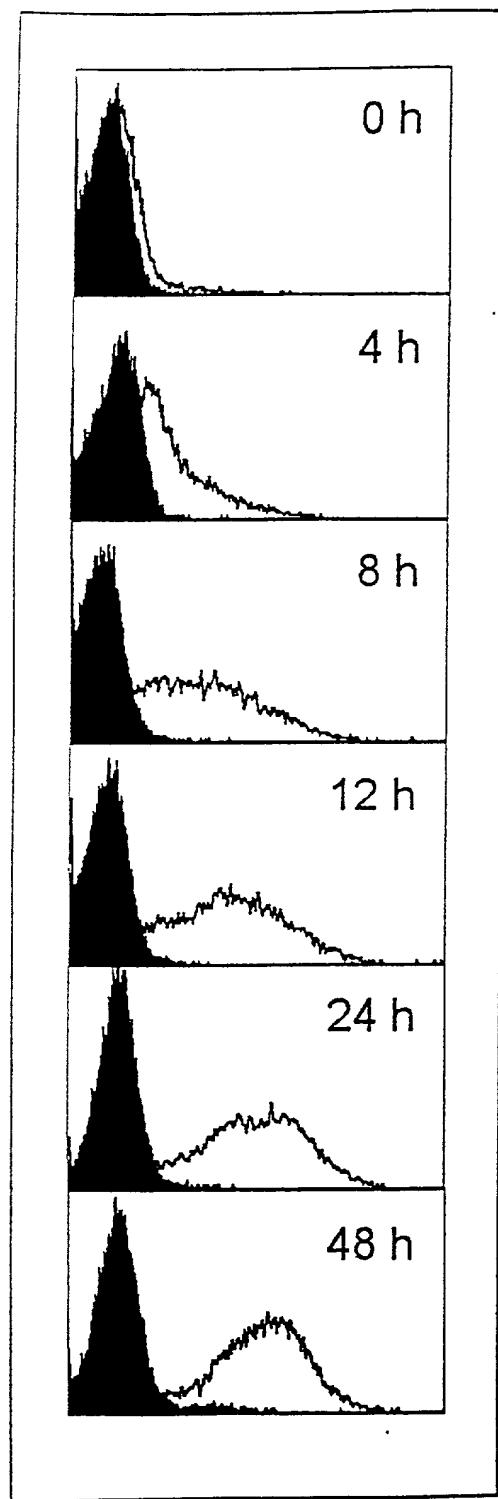


FIG. 2a

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**FIG. 2b**

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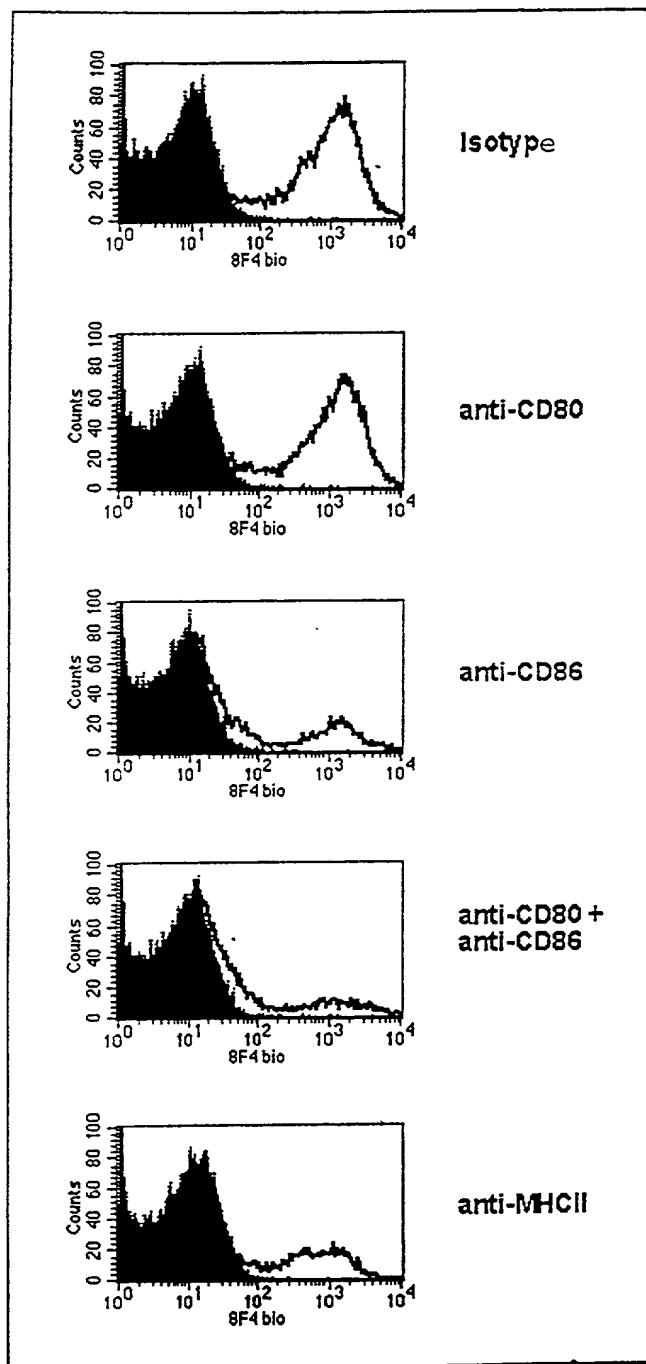


FIG. 3

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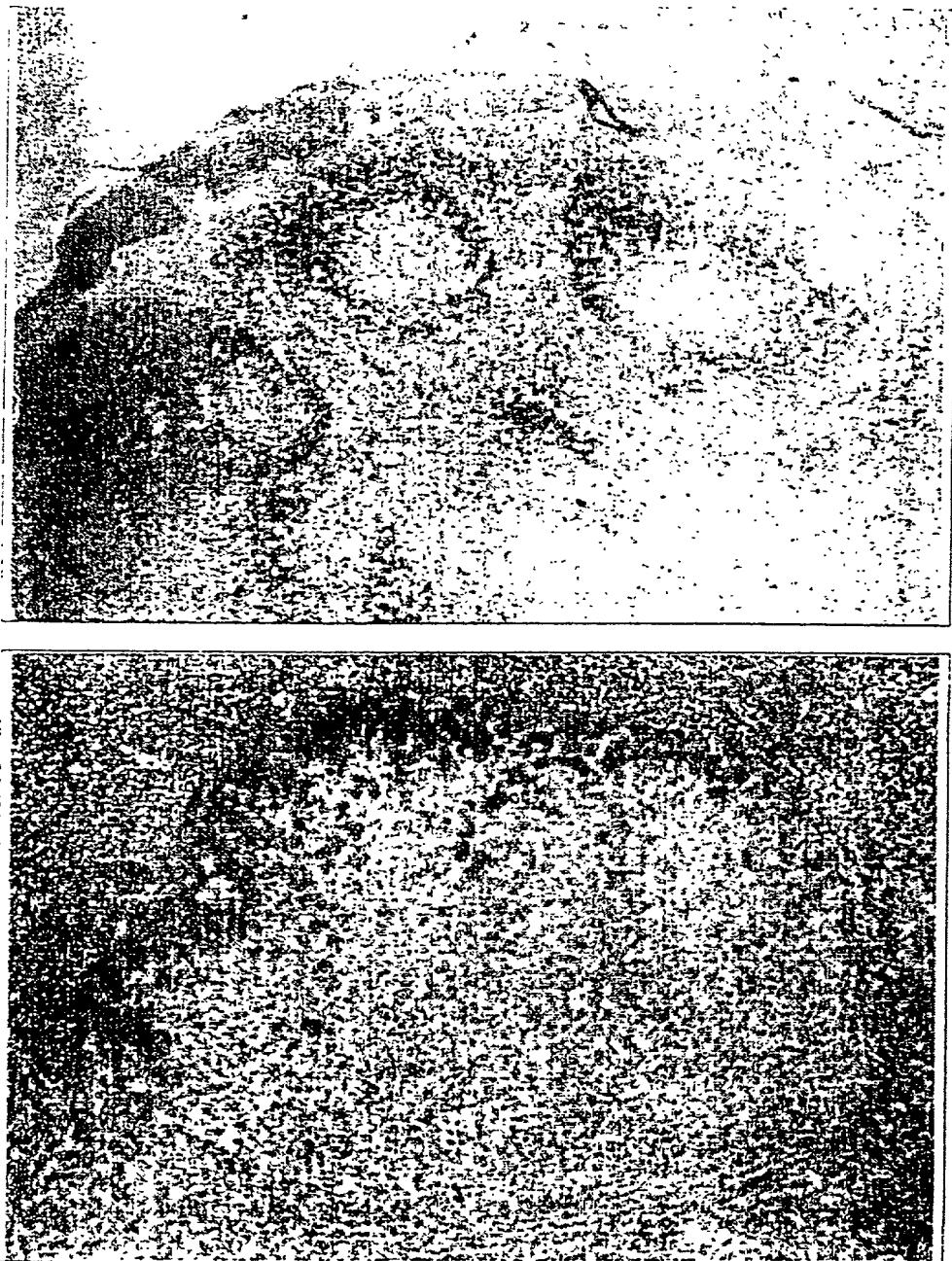


FIG. 4

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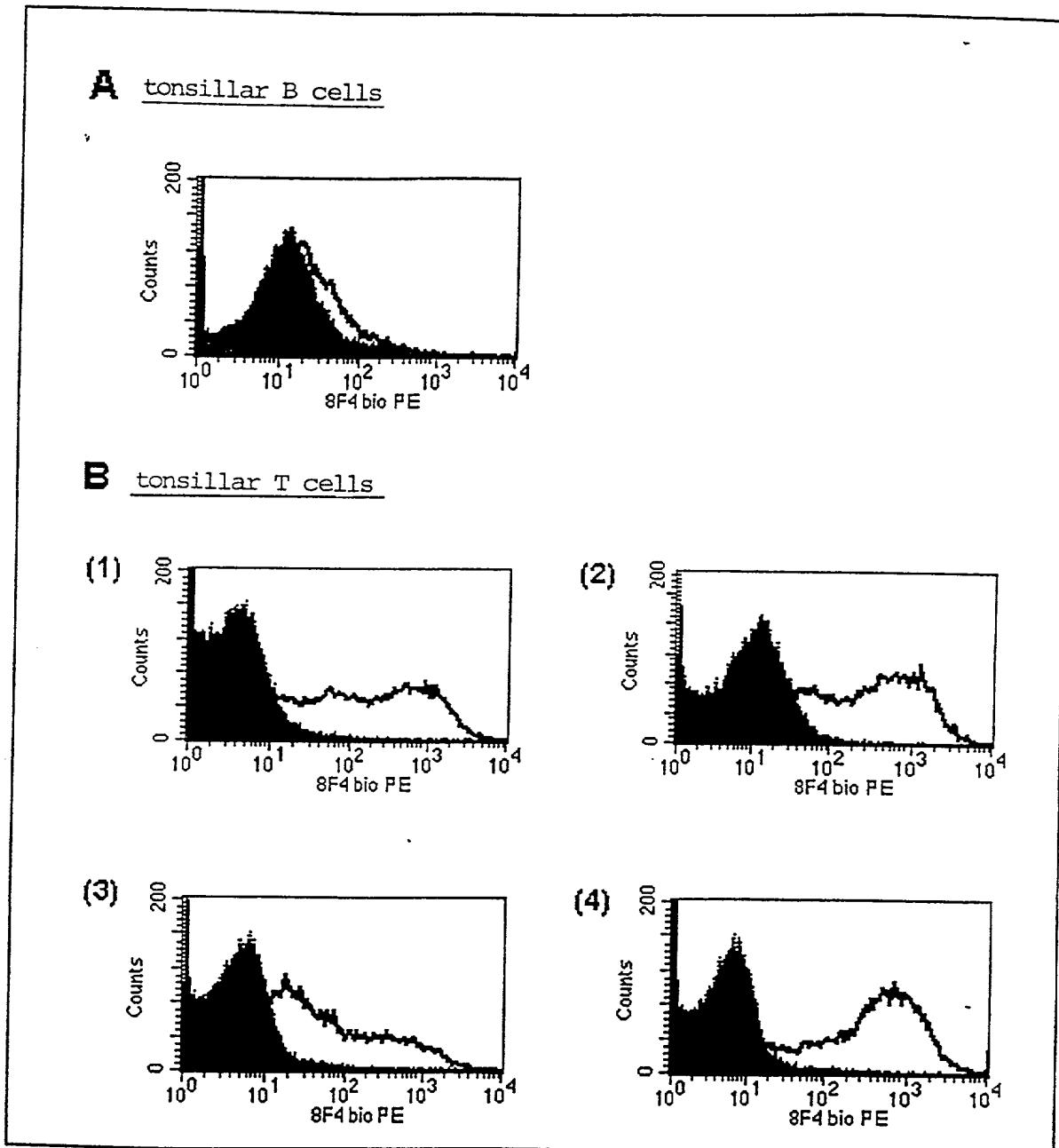


FIG. 5

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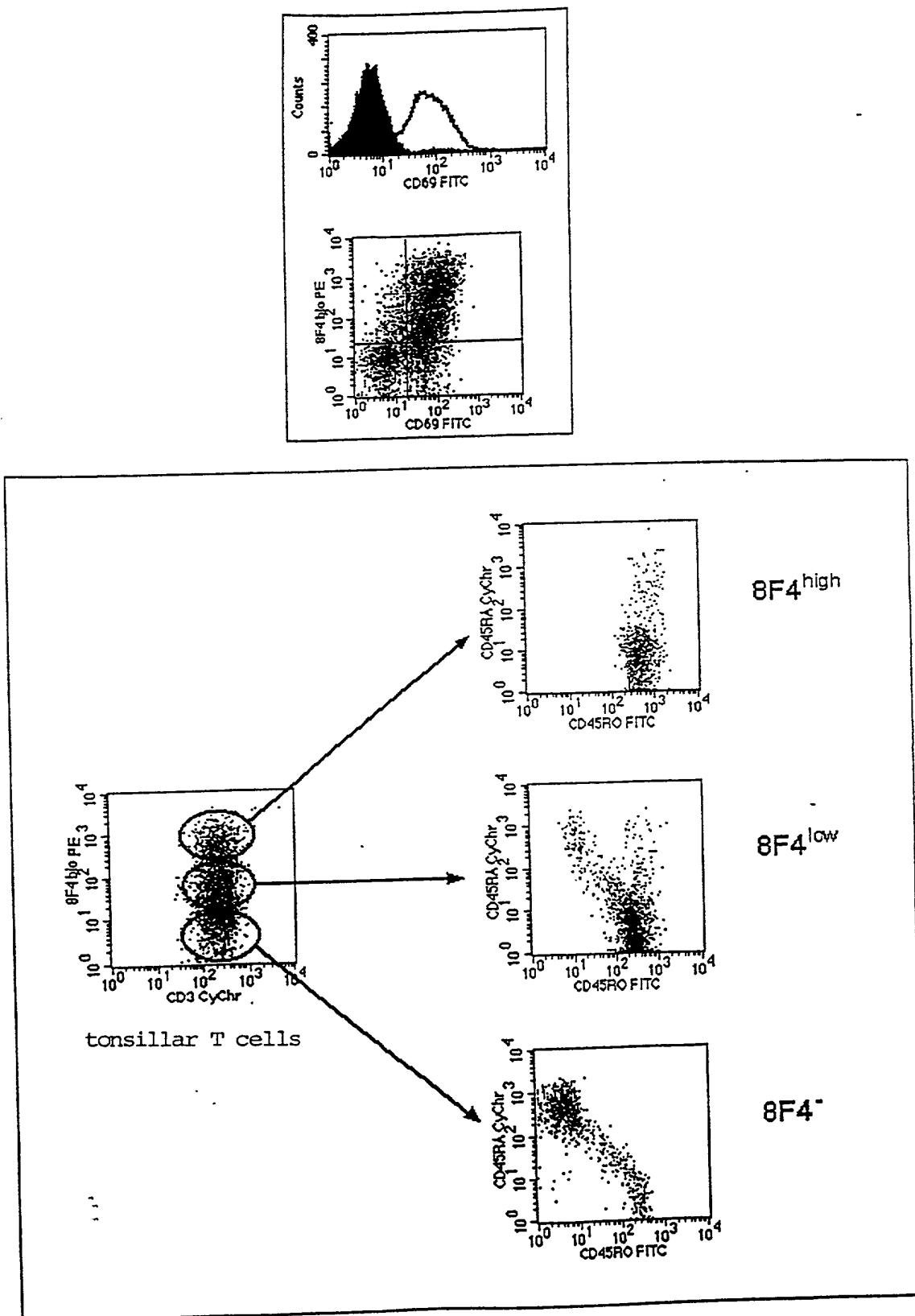


FIG. 6

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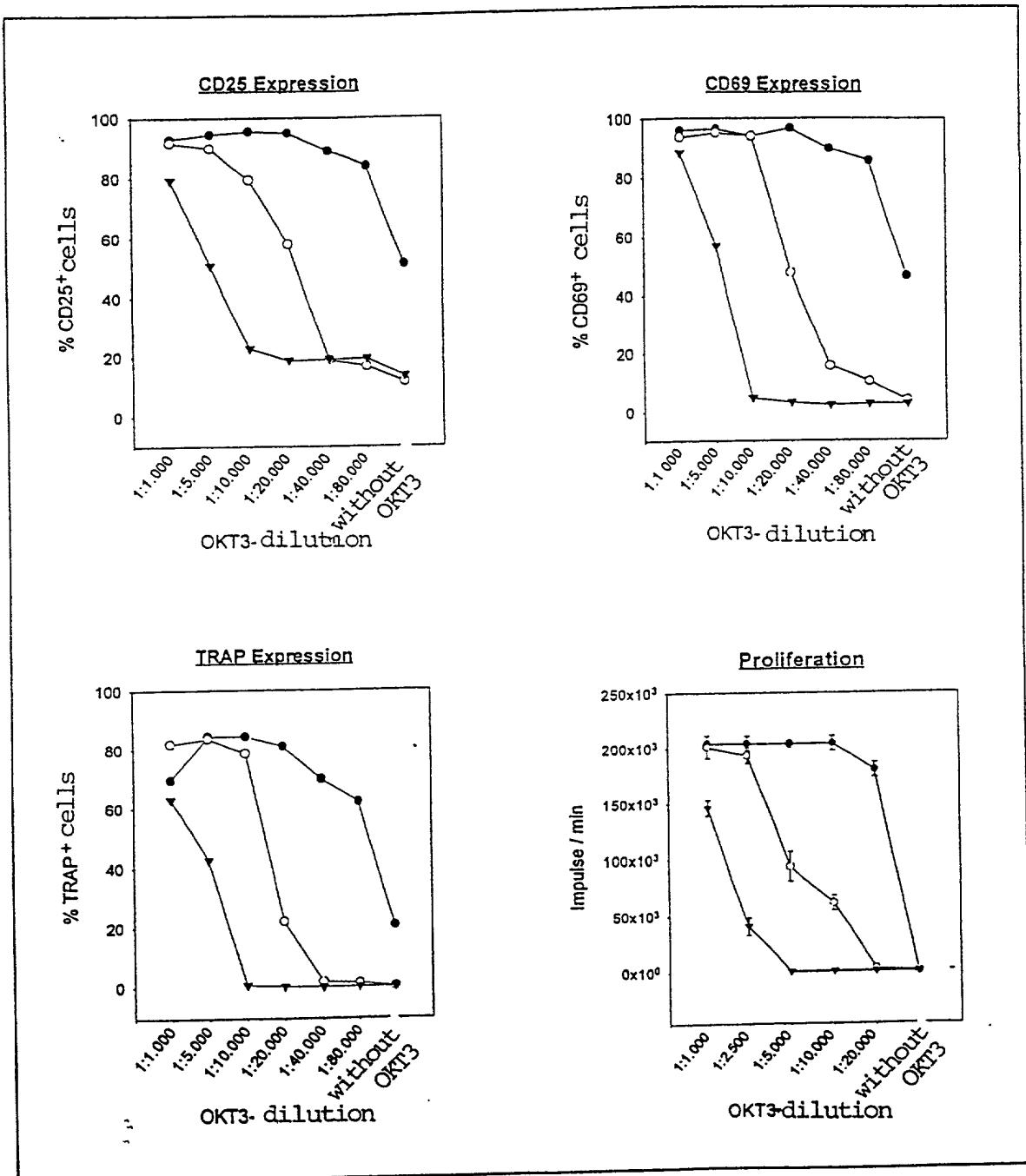


FIG. 7

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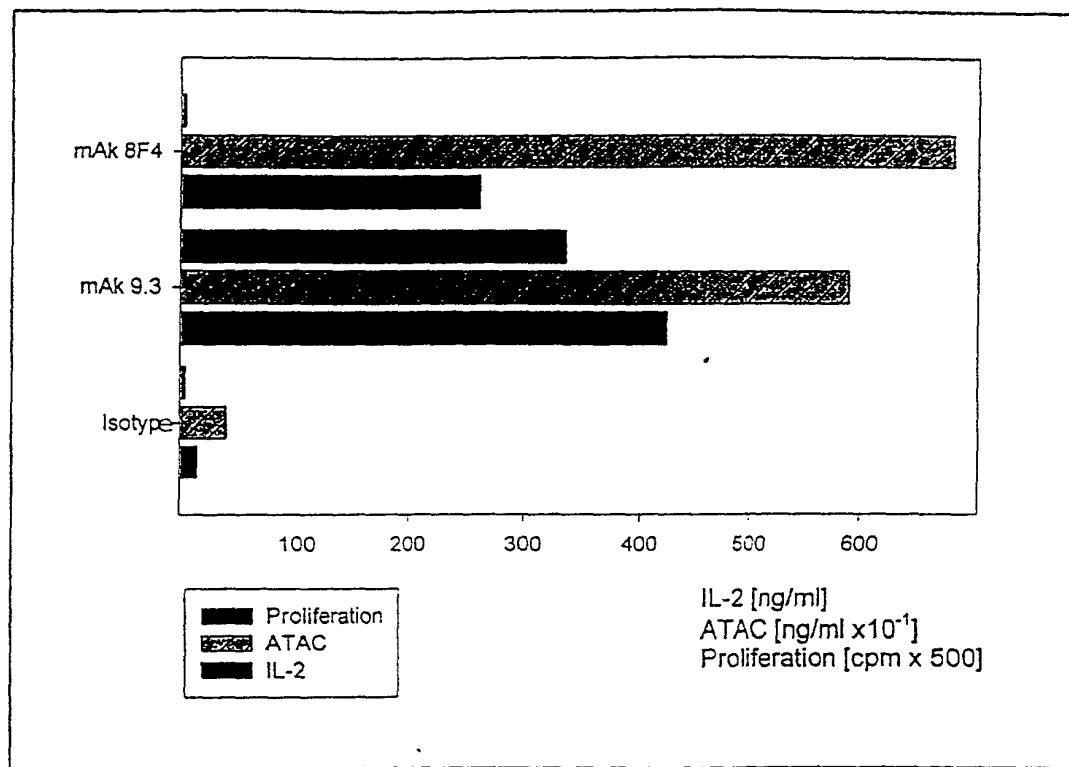


FIG. 8

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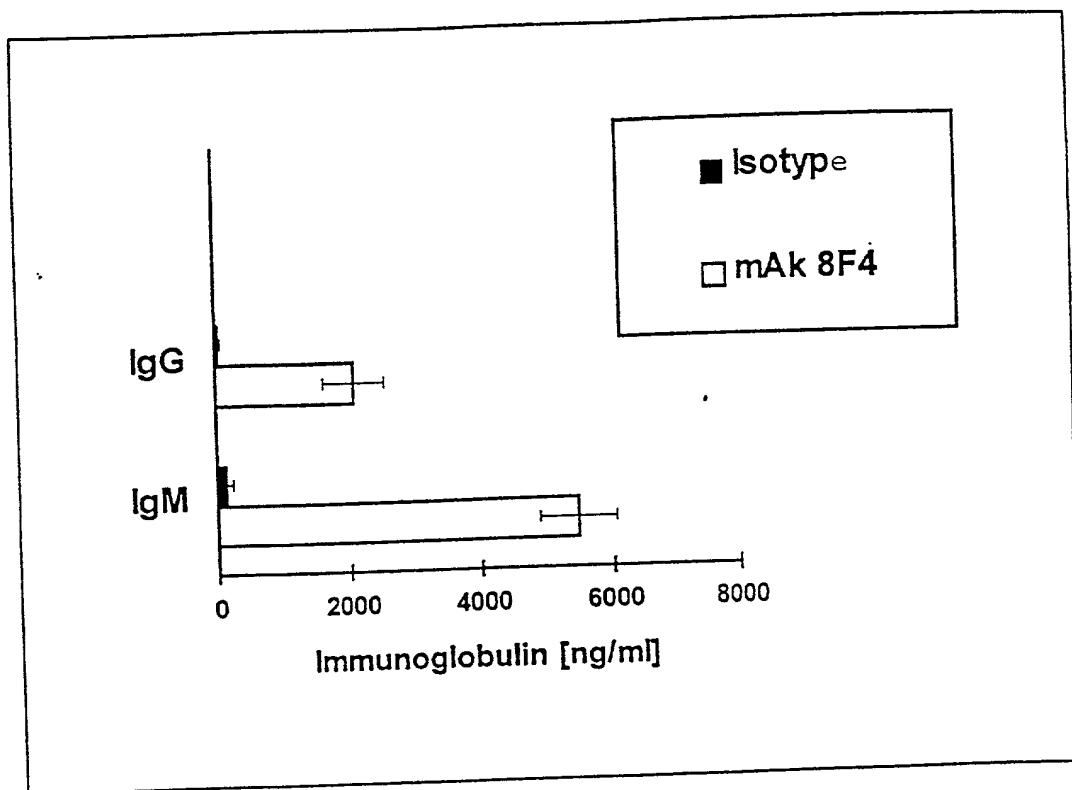


FIG. 9

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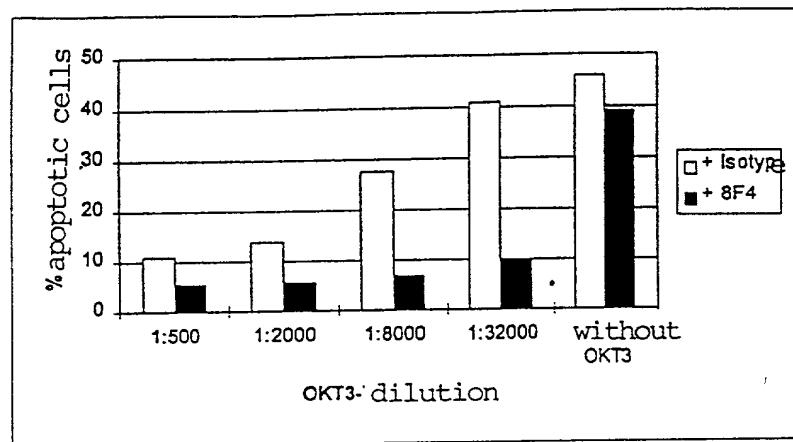


FIG. 10

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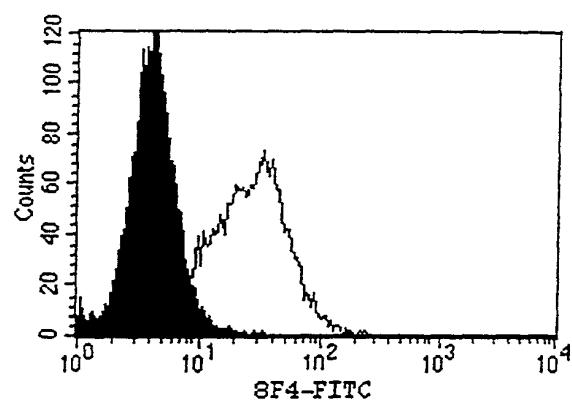


FIG. 11

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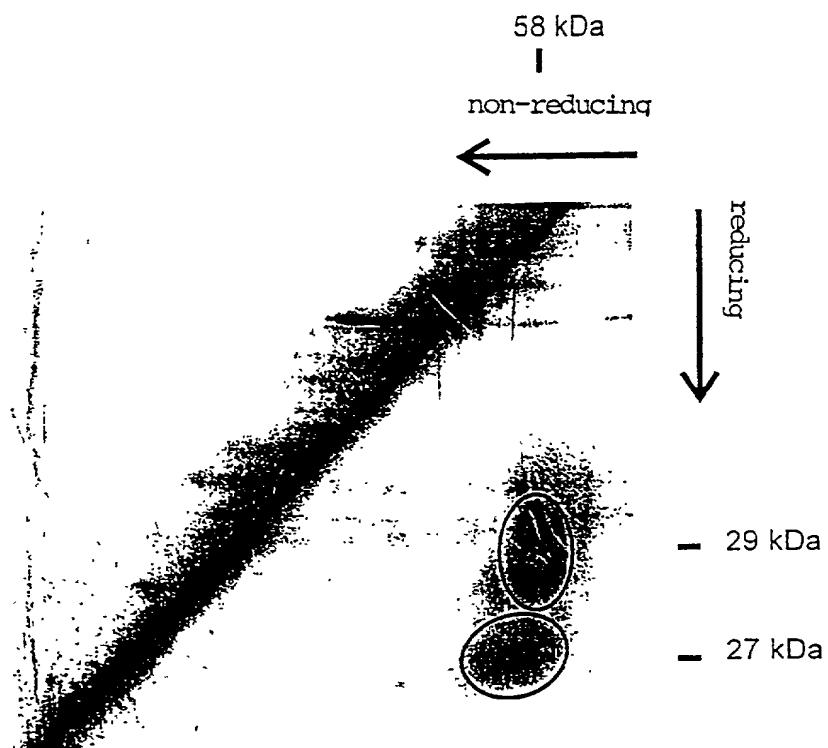


FIG. 12

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FIG. 13

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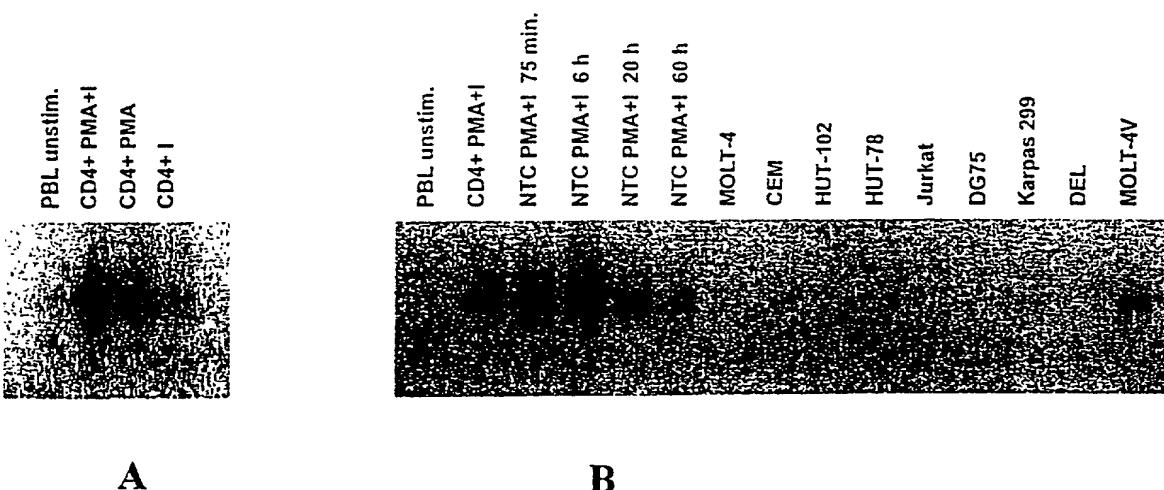


FIG. 14

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FDPPPFKVTLTGGYLHIYESQLCCQLKFWLPIGCAAFVVVCILGCILICWLTKKKYS
SSVHDPNGEYMFMRAVNTAKKSRLTDVTL

FIG. 15

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CGAGAGCCTGAATTCACTGTCAGCTTGAACACTGAACGCGAGGACTGTTAAGTGTCT
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G

FIG. 16



PATENT

ATTORNEY DOCKET NO: 50125/011001

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled COSTIMULATING POLYPEPTIDE OF T CELLS, MONOCLONAL ANTIBODIES, AND THE PREPARATION AND USE THEREOF the specification of which

is attached hereto.

was filed on March 22, 2000 as Application Serial No. 09/509,283
and was amended on _____.

was described and claimed in PCT International Application No. _____
filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

FOREIGN PRIORITY RIGHTS: I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Serial Number	Filing Date	Priority Claimed?
Germany	19741929.1	September 23, 1997	Yes
Germany	19821060.4	May 11, 1998	Yes

PROVISIONAL PRIORITY RIGHTS: I hereby claim priority benefits under Title 35, United States Code, §119(e) and §120 of any United States provisional patent application(s) listed below filed by an inventor or inventors on the same subject matter as the present application and having a filing date before that of the application(s) of which priority is claimed:

Serial Number	Filing Date	Status



COMBINED DECLARATION AND POWER OF ATTORNEY

NON-PROVISIONAL PRIORITY RIGHTS: I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Serial Number	Filing Date	Status

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Paul T. Clark, Reg. No. 30,162, Karen L. Elbing, Ph.D. Reg. No. 35,238, Kristina Bleker-Brady, Ph.D. Reg. No. 39,109, Susan M. Michaud, Ph.D. Reg. No. 42,885, Mary Rose Scozzafava, Ph.D., Reg. No. 36,268, James D. DeCamp, Ph.D., Reg. No. 43,580, Sean J. Edman, Reg. No. 42,506.

Address all telephone calls to: Karen L. Elbing, Ph.D. at 617/428-0200.

Address all correspondence to: Karen L. Elbing, Ph.D. at Clark & Elbing LLP, 176 Federal Street, Boston, MA 02110.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full Name (First, Middle, Last)	Residence Address (City, State, Country)	Post Office Address (Street, City, State, Country)	Citizenship
<u>Richard Kroczek</u>	Dickens-Weg 56 D-14055 Berlin	Dickens-Weg 56 D-14055 Berlin <i>DE</i>	Germany
Signature: <i>R. Kroczek</i>			Date: <u>6/28/00</u>